

Kuzbanian Controls Proteolytic Processing of Notch and Mediates Lateral Inhibition during *Drosophila* and Vertebrate Neurogenesis

Duoja Pan and Gerald M. Rubin
Howard Hughes Medical Institute
Department of Molecular and Cell Biology
University of California
Berkeley, California 94720-3200

Summary

Notch and the disintegrin metalloprotease encoded by the *kuzbanian* (*kuz*) gene are both required for a lateral inhibition process during *Drosophila* neurogenesis. We show that a mutant KUZ protein lacking protease activity acts as a dominant-negative form in *Drosophila*. Expression of such a dominant-negative KUZ protein can perturb lateral inhibition in *Xenopus*, leading to the overproduction of primary neurons. This suggests an evolutionarily conserved role for KUZ. The Notch family of receptors are known to be processed into smaller forms under normal physiological conditions. We provide genetic and biochemical evidence that Notch is an *in vivo* substrate for the KUZ protease, and that this cleavage may be part of the normal biosynthesis of functional Notch proteins.

Introduction

Cell–cell interactions play an important role in regulating cell fate decisions and pattern formation during the development of multicellular organisms. One of the evolutionarily conserved pathways that plays a central role in local cell interactions is mediated by the transmembrane receptors encoded by the *Notch* (*N*) gene of *Drosophila*, the *lin-12* and *glp-1* genes of *C. elegans*, and their vertebrate homologs (reviewed in Artavanis-Tsakonas et al., 1995). For simplicity, these proteins will be referred to as Notch proteins throughout this paper.

Several lines of evidence suggest that the proteolytic processing of Notch proteins is important for their function. In addition to the full-length proteins, antibodies against the intracellular domains of Notch proteins have detected C-terminal fragments of 100–120 kDa (hereafter referred to as 100 kDa fragments) in *Drosophila* (Fehon et al., 1990), *C. elegans* (Crittenden et al., 1994), and mammalian cells (Aster et al., 1994; Zagouras et al., 1995). Pulse–chase experiments (Aster et al., 1994; Zagouras et al., 1995) suggest that the 100 kDa fragment is generated from the full-length protein by proteolytic cleavage, which presumably generates an N- and a C-terminal fragment. Antibodies against the extracellular region of the *C. elegans* GLP-1 can detect the N-terminal fragment, which appears to remain associated with the C-terminal fragment (Crittenden et al., 1994). While studies on vertebrate Notch proteins suggest that this cleavage occurs in the extracellular domain close to the transmembrane domain (Aster et al., 1994; Kopan et al., 1996), the protease responsible for the processing and the function of the processed forms, which can comprise the majority of the Notch proteins

in certain cells (Zagouras et al., 1995), are unknown. In particular, it is not clear whether the full-length Notch proteins or the proteolytically cleaved forms are the functional receptors.

Engineered forms of Notch proteins that delete the extracellular domain or both the transmembrane and the extracellular domains encode constitutively active receptors, and in some instances are found to be localized in the nucleus (reviewed in Artavanis-Tsakonas et al., 1995). These observations led to the hypothesis that upon ligand activation, Notch is proteolytically processed to release its cytoplasmic domain, which then migrates to the nucleus to control the transcription of target genes (see for example, Kopan et al., 1996). However, it is not clear from the available data whether any particular processing event of the Notch proteins detected *in vivo* is simply a normal part of the biosynthesis of a functional receptor or a consequence of receptor activation upon ligand binding. Furthermore, Notch proteins have not been detected in the nucleus under normal physiological conditions (reviewed in Artavanis-Tsakonas et al., 1995). Thus the mechanism(s) of Notch activation are still largely unknown.

During *Drosophila* neurogenesis, a single neural precursor is singled out from a group of equivalent cells through a lateral inhibition process in which the emerging neural precursor cell prevents its neighbors from taking on the same fate (reviewed in Simpson, 1990). Genetic studies in *Drosophila* have implicated a group of “neurogenic genes” including *N* in lateral inhibition. Loss-of-function mutations in any of the neurogenic genes result in hypertrophy of neural cells at the expense of epidermis (reviewed in Campos-Ortega, 1993). Recently we identified a new neurogenic gene, *kuzbanian* (*kuz*) (Rooke et al., 1996). Clonal analyses revealed that *kuz* (Rooke et al., 1996), like *N* (Heitzler and Simpson, 1991), is required cell autonomously for cells to be inhibited from a neural fate, indicating that KUZ and Notch function in the same cell.

KUZ belongs to the recently defined ADAM family of transmembrane proteins, members of which contain both a disintegrin and metalloprotease domain. This is an emerging gene family that in the mouse genome consists of at least 20 members (reviewed in Wolfsberg et al., 1995). The prototypes of the ADAM family, fertilin α and β (also called PH-30 α and β), are sperm surface molecules implicated in sperm–egg binding during fertilization (Blobel et al., 1992). Another ADAM protein, meltrin, has been implicated in myoblast fusion (Yagami-Hiromasa et al., 1995). More recently, an ADAM protein was identified as a TNF- α converting enzyme (TACE) that is responsible for releasing TNF- α from the cell surface by proteolytic cleavage of a transmembrane precursor (Black et al., 1997; Moss et al., 1997).

In this study, we have conducted a series of experiments in both *Drosophila* and *Xenopus* in order to understand the molecular mechanisms of KUZ function. We have engineered various mutant forms of KUZ protein and assayed their activities in transgenic flies. We found that a mutant KUZ protein lacking protease activity interferes with the endogenous KUZ activity and functions as

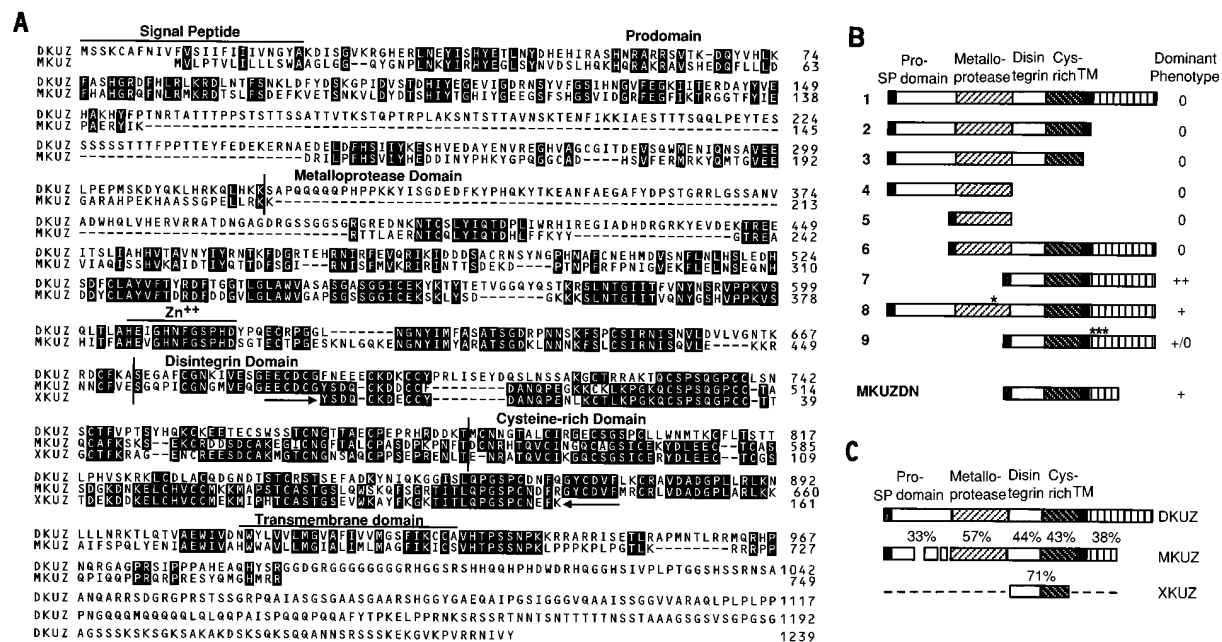


Figure 1. Alignment of Predicted KUZ Protein Sequences from Different Species and Summary of Constructs Tested in This Study

(A) Sequence alignment of predicted KUZ proteins from *Drosophila* (DKUZ), mouse (MKUZ), and *Xenopus* (XKUZ). The full-length amino acid sequence of MKUZ was deduced from the nucleotide sequence of two overlapping cDNA clones. Partial amino acid sequence of XKUZ was deduced from the nucleotide sequence of a PCR product that includes parts of the disintegrin and Cys-rich domains. The alignments were produced using Geneworks software (IntelliGenetics). Residues identical among two species are highlighted. Predicted functional domains are indicated. Amino acid sequences from which degenerate PCR primers were designed are indicated with arrows. Orthologs of *kuz* are also present in *C. elegans* (GenBank accession nos. D68061 and M79534), rat (Z48444), bovine (Z21961), and human (Z48579).

(B) Summary of constructs used in this study and their overexpression phenotypes. Different domains are indicated by shadings. Asterisks indicate where point mutations were introduced. Constructs 1–9 are based on DKUZ, while MKUZDN is based on MKUZ. Abbreviations: (++) , strong phenotype; (+) , weak phenotype; (0) , no phenotype.

(C) Schematic diagram of DKUZ, MKUZ, and XKUZ. The percentages given refer to sequence identity in the indicated domains between MKUZ and either DKUZ or XKUZ.

a dominant-negative form, suggesting that the protease activity of KUZ is essential to its biological functions. To extend our analyses of KUZ functions into vertebrates, cDNAs encoding a mouse KUZ homolog (*mkuz*) were isolated. A dominant-negative form of MKUZ (MKUZDN) was created that when overexpressed in *Drosophila*, resulted in phenotypes similar to those created by its *Drosophila* counterpart. We further show that MKUZDN can perturb lateral inhibition during *Xenopus* neurogenesis and results in the overproduction of primary neurons. These findings suggest that KUZ plays an evolutionarily conserved role during lateral inhibition. Since the products of both *N* and *kuz* genes are involved in lateral inhibition during neurogenesis, we investigated their genetic interactions. These studies suggest that *kuz* acts genetically upstream of *N*. Together with our previous clonal analyses indicating that *kuz* and *N* function in the same cell during lateral inhibition and our finding that the protease activity is essential to KUZ function, the genetic interaction between *kuz* and *N* prompted us to investigate the possibility that KUZ might be involved in the proteolytic processing of Notch. We find that while Notch is proteolytically processed in wild-type embryos to generate the 100 kDa species, this processing fails to occur in *kuz* mutant embryos. Similarly, expression of a dominant-negative form of KUZ in imaginal discs or tissue culture cells blocks

Notch processing. Our data suggest a model in which the primary Notch translation product is proteolytically cleaved by KUZ as part of the normal biosynthesis of a functional Notch protein.

Results

Deletion of the Metalloprotease Domain of KUZ Results in a Dominant-Negative Form

kuz encodes a transmembrane protein with multiple domains (Figure 1). To investigate how the different domains of KUZ contribute to its biological functions, full-length and various N- and C-terminal truncations of KUZ were generated (constructs 1–4 and 7, Figure 1B) and expressed under the pGMR vector (Hay et al., 1994) in the developing retina of *Drosophila*. One of these truncations (7), which is missing the protease domain, resulted in a dominant rough eye phenotype (data not shown), while the others failed to produce any dominant phenotypes. To examine the phenotypes produced by this truncation in other tissues, we expressed KUZ truncations using the pDMR vector, which contains the *decapentaplegic* (*dpp*) disc-specific enhancer element (see Experimental Procedures) that drives gene expression in several tissues including parts of the notum and the wing blade, two tissues that are known to be affected in *kuz* mutant clones. While the expression of full-length

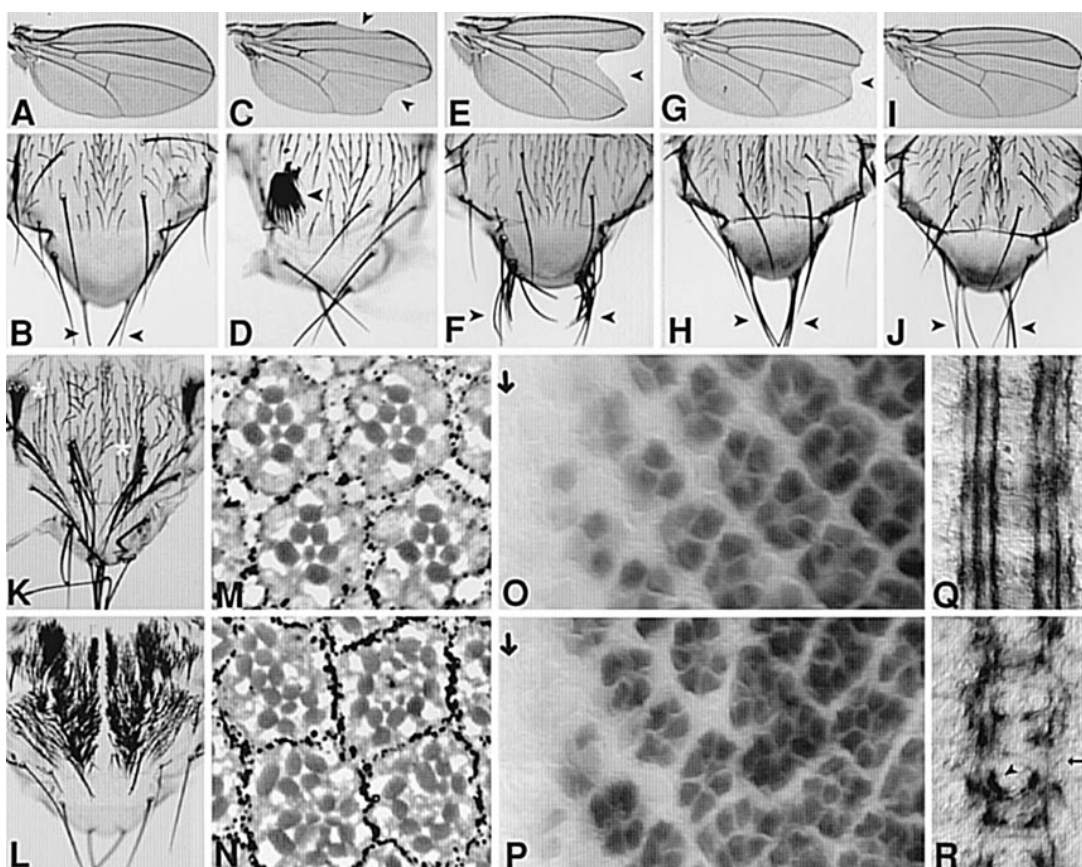


Figure 2. Expression of Dominant-Negative Forms of KUZ Resulted in Mutant Phenotypes in *Drosophila*

(A) and (B) show wild-type wing and notum morphology, respectively. Note the presence of larger macrochaetes and smaller microchaetes on the notum. Posterior scutellar bristles are indicated with arrowheads. Single bristles are found at these positions. (C) and (D) show a wing and a notum bearing *kuz* mutant clones. *kuz* mutant clones reaching the wing margin result in notches on the wing blade (arrowheads in [C]), while mutant clones in the notum result in supernumerary bristles (arrowhead in [D]). (E) and (F) show the wing and the notum from a transgenic fly line expressing KUZDN (construct 7 in Figure 1B) under pDMR. Note the presence of a notch along the anterior/posterior compartment border of the adult wing (E) and supernumerary scutellar bristles on the notum (arrowheads in [F]). These phenotypes are similar to those seen in *kuz* mutant clones (see [C] and [D]). The positions at which mutant phenotypes are observed in (E) and (F) closely match the expression pattern of *dpp* in the wing imaginal disc. (G) and (H) show the wing and the notum from a transgenic fly line expressing a dominant-negative MKUZ under pDMR. This construct (MKUDN-1) contains the signal peptide from *Drosophila* KUZ fused to a truncation of MKUZ that removed sequences N-terminal to the disintegrin domain. Note the presence of wing notch (arrowhead in [G]) and extra scutellar bristles (arrowheads in [H]). (I) and (J) show the wing and notum from a transgenic fly line expressing a full-length KUZ construct containing a point mutation in the protease active site (E606A) under pDMR. Note the presence of wing notch (arrowhead in [I]) and extra scutellar bristles (arrowheads in [J]). (K) and (L) show notums from flies containing a heat-inducible KUZDN transgene and subjected to a 60 min heat shock at 37°C in the third larval instar (K) or at 5 hr APF (L). Note the presence of extra macrochaetes in (K) (marked by asterisks), and extra microchaetes in (L) (compare [K] and [L] to the wild-type notum in [B]). (M) and (N) show sections of adult eyes from wild type and a fly line carrying the *rough*/KUZDN transgene, respectively. While sections of a wild-type eye reveal 7 photoreceptors per ommatidium at this level (M), those of *rough*/KUZDN reveal an average of 13 photoreceptors (N). (O) and (P) show the expression of ELAV, a neuronal cell marker, in the eye imaginal discs from wild-type and *rough*/KUZDN flies, respectively. The arrow marks the position of the morphogenetic furrow. In wild-type eye discs, photoreceptors differentiate in a highly ordered and stereotyped manner (O). In *rough*/KUZDN eye discs, extra ELAV positive cells are recruited into developing ommatidia (P). (Q) and (R) show the morphology of CNS axonal tracts revealed by anti-Fas II MAb 1D4 in wild-type embryos and embryos expressing KUZDN in all neurons, respectively. Note the break of longitudinal tracts (arrow) and the stalling of axons (arrowhead) in embryos expressing KUZDN (R). Overexpression of KUZDN was achieved by crossing flies carrying UAS/KUZDN to flies bearing ELAV-GAL4.

KUZ did not result in dominant phenotypes (data not shown), expression of construct 7 under pDMR resulted in supernumerary bristles on the notums and notches on the wing blades (Figures 2E and 2F). These phenotypes resemble those seen in somatic clones homozygous for *kuz* loss-of-function mutations (Figures 2C and 2D), suggesting that this construct functions in a dominant-negative manner by interfering with endogenous *kuz*

activity. Supporting this hypothesis, we observed that the mutant phenotypes resulting from this construct are dominantly enhanced by removing one copy of the endogenous *kuz* gene; that is, the phenotypes of *kuz*^{+/−} individuals carrying this construct are more severe than those of *+/+* individuals. Conversely, additional wild-type KUZ protein from a transgene expressing full-length KUZ suppresses these phenotypes (data not

shown). We will therefore refer to construct 7 as KUZDN (KUZ dominant-negative).

Although the structure of KUZDN suggests that its dominant-negative activity results from the loss of protease activity, it is conceivable that the approximately 600 amino acids deleted in KUZDN might affect other activities important for KUZ functions. To directly address the functional relevance of the protease domain, we introduced into full-length KUZ a point mutation (E606 to A) in the putative zinc binding site (Figure 1A) of the protease domain. This glutamate is thought to be a catalytic residue and is absolutely conserved among all known metalloproteases (Jiang and Bond, 1992). Thus, this point mutation should abolish protease activity while having minimal impact on the other activities of KUZ. Consistent with this hypothesis, overexpression of KUZ^{E606A} (construct 8 in Figure 1B) gave similar, although somewhat weaker, dominant phenotypes (Figures 2I and 2J) to those seen with KUZDN (Figures 2E and 2F).

KUZDN Interferes with Lateral Inhibition during Bristle Development

The notums of *Drosophila* adults carry two types of sensory bristles, macrochaetes and microchaetes. The sensory organ precursor cells (SOPs) that generate the macrochaetes are selected from pools of equivalent cells by lateral inhibition mostly during the third instar larval stage, while the SOPs for the microchaetes are singled out during the early pupae stage (Hartenstein and Posakony, 1989; Huang et al., 1991). *N* is required for this process, and removal of *N* function at larval and pupal stages differentially affects these two types of bristles (Hartenstein and Posakony, 1990). If KUZ is required for lateral inhibition, we would expect to generate similar phenotypes by expressing KUZDN at these times. We generated flies containing KUZDN under the control of the *hsp70* promoter, and applied one hour heat pulses at various times during larval and pupal development. We observed that while heat pulses applied during third instar larval stage resulted in supernumerary macrochaetes only (Figure 2K), heat pulses applied during early pupal stages (0–13 hrs after puparium formation [APF]) resulted in supernumerary microchaetes only (Figure 2L), similar to the phenotypes that resulted from removing *N* function at these times using a temperature-sensitive *N* allele (Hartenstein and Posakony, 1990). These time points match the periods when SOPs for each bristle type are selected from pools of equivalent cells (Hartenstein and Posakony, 1989; Huang et al., 1991), suggesting that KUZDN interferes with lateral inhibition during the selection of SOPs.

Expression of KUZDN Results in Mutant Phenotypes in Other Tissues

kuz mutant clones are known to affect other tissues such as the eye. Analysis of *kuz* mutant phenotypes in the eye was complicated by the gross structural abnormalities associated with loss-of-function mutant clones (Rooke et al., 1996). KUZDN offered us a useful tool to perturb *kuz* functions in a more restricted manner. Neurogenesis in the developing eye initiates within the

morphogenetic furrow and proceeds in a stepwise manner. Initially, R8 is singled out from a group of equivalent cells. Following that, R2/R5, R3/R4, R1/R6, and R7 are added to each ommatidial cluster (reviewed in Wolff and Ready, 1993). We perturbed *kuz* functions by expressing KUZDN under the control of the *rough* enhancer, which drives gene expression in all cells within the morphogenetic furrow as well as transiently in R2, R5, R3, and R4 posterior to the furrow (Heberlein et al., 1994). Flies carrying the *rough*/KUZDN transgene had supernumerary photoreceptor cells in each ommatidium (compare Figure 2N with 2M). Neuronal differentiation in these transgenic flies was followed by staining for ELAV, a neuronal marker, in eye imaginal discs. Consistent with the adult eye phenotype, we observed the recruitment of extra neurons into each ommatidial cluster in the developing retina (compare Figure 2P with 2O). These experiments suggested that *kuz* function is required to limit the number of photoreceptor neurons recruited into each ommatidium.

Besides its functions in determining neural fate, *kuz* is also required for axonal extension at later stages of neural development (Fambrough et al., 1996). The question arises whether these defects are due to the lack of *kuz* functions in the axons themselves or in the cells contributing to the substrate upon which the axons extend. To distinguish these alternatives, we expressed KUZDN under the control of the ELAV promoter using the GAL4-UAS system (Brand and Perrimon, 1993). The ELAV promoter drives gene expression in maturing and mature neurons, but not neuroblasts, thus allowing one to bypass the requirement for *kuz* in neural fate determination. We observed that embryos expressing KUZDN in developing neurons show major defects in axonal pathways, such as disruption of longitudinal axonal tracts (compare Figure 2R with 2Q). In general, this phenotype is similar to that observed in zygotic *kuz* mutant embryos (Fambrough et al., 1996), supporting the hypothesis that KUZ provides a proteolytic activity synthesized by axons and required by them to extend growth cones through the extracellular matrix.

Evolutionary Conservation of *kuz* Structure and Function

Database searches revealed sequences representing putative *kuz* orthologs in *C. elegans*, rat, bovine, and human. The bovine homolog was initially isolated as a proteolytic activity on myelin basic protein in vitro (Howard et al., 1996), but its in vivo function remains unknown. As a first step in understanding the potential functions of *kuz* in vertebrate neurogenesis, we isolated and sequenced cDNAs representing a full-length mouse *kuz* homolog. This mouse protein (MKUZ) is 45% identical in primary sequence with *Drosophila* KUZ (DKUZ, Figure 1), and 95% identical with the bovine protein (not shown). Sequence similarity between MKUZ and DKUZ extends over the whole coding region, except that MKUZ, like other vertebrate KUZ homologs, has a much shorter intracellular domain. The intracellular domain of MKUZ contains a stretch of 9 amino acid residues (934–942) that are absolutely conserved with DKUZ. To determine the functional importance of this sequence

similarity, we introduced into KUZDN mutations in several conserved residues in this region (936TPSS939 to AAAA; construct 9 in Figure 1B). These mutations dramatically reduced KUZDN activity (data not shown), implying that the altered residues are important for KUZ function. However, additional experiments will be required to determine if it is the expression, subcellular localization, or activity of KUZDN that is disrupted.

Based on the structure of KUZDN described above, we engineered a putative dominant-negative form of MKUZ (MKUZDN, Figure 1B); that is, an MKUZ construct that is missing the protease domain. When overexpressed in *Drosophila* using the pDMR vector, MKUZDN resulted in dominant phenotypes (Figures 2G and 2H) resembling those created by its *Drosophila* counterpart (Figures 2E and 2F). To test more directly the involvement of MKUZ in vertebrate neurogenesis, we injected *in vitro* transcribed mRNA encoding MKUZDN into *Xenopus* embryos. Primary neurons in *Xenopus* are generated in precise and simple patterns and can be identified by their expression of a neural-specific β -tubulin gene (*N-tubulin*). This assay has been used previously to demonstrate a conserved role for certain neurogenic genes in singling out primary neurons in *Xenopus* by lateral inhibition (Chitnis et al., 1995). If a *kuz*-like activity is required for the lateral inhibition process in *Xenopus*, we would expect interference with this endogenous *kuz* activity to result in the overproduction of primary neurons. Indeed, injection of mRNA encoding MKUZDN resulted in extra *N-tubulin* positive cells (Figures 3A and 3B). Consistent with the notion that *kuz* acts to limit the number of cells that differentiate as neurons from a group of competent cells, these extra *N-tubulin* positive cells were confined to domains of primary neurogenesis and were not observed at ectopic positions.

To provide further evidence for an endogenous *kuz* activity during primary neurogenesis in *Xenopus*, we examined the expression pattern of a *Xenopus kuz* homolog (*Xkuz*). A cDNA fragment representing a portion of *Xkuz* (Figure 1) was isolated (see Experimental Procedures) and used to generate RNA probes for *in situ* hybridization under high stringency. *Xkuz* is expressed uniformly in gastrulating and neural plate stage embryos, including the domains of primary neurogenesis (Figures 3C and 3D). In older embryos, *Xkuz* continues to be widely expressed, with an elevated level in neural tissues (Figures 3E and 3F). Thus, *Xkuz* is expressed at the appropriate time and place for a potential role in primary neurogenesis in *Xenopus*.

kuz Acts Genetically Upstream of *N* in Neurogenesis

Notch is known to play a central role in lateral inhibition and acts as a receptor for the lateral inhibition signal (reviewed in Simpson, 1990; Campos-Ortega, 1993; Artavanis-Tsakonas et al., 1995). Clonal analyses revealed that *kuz*, like *N*, is required cell autonomously for cells to be inhibited from a neural fate and is thus likely to act in the same cell where *N* function is required (Rooke et al., 1996). Since loss-of-function mutations of both genes exhibit similar neurogenic phenotypes, we sought to determine their order of action by examining the phenotype produced by combining a gain-of-function *N* mutant and a loss-of-function *kuz* mutant. Expression of

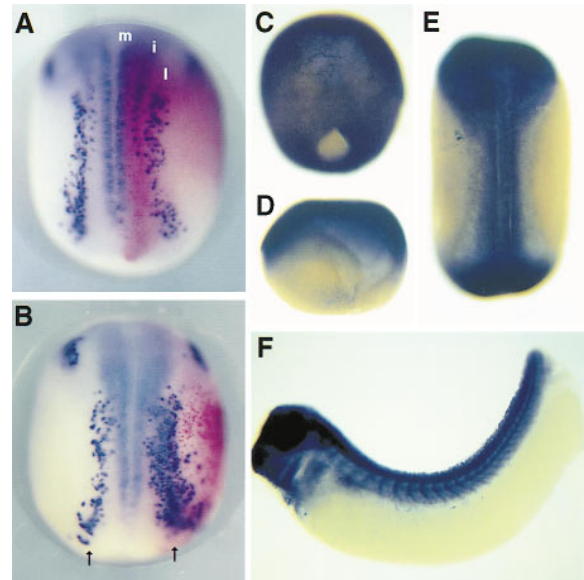


Figure 3. Injection of mRNA Encoding MKUZDN Leads to Supernumerary Primary Neurons in *Xenopus* Embryos and the Expression Pattern of a *Xenopus kuz* Gene

(A) and (B) show whole-mount *Xenopus* embryos at the neural plate stage hybridized with an *N-tubulin* probe. Normally, three stripes of primary neurons, medial (m), intermediate (i), and lateral (l), are present on each side of the neural plate (A). RNA encoding *lacZ* alone (A) or RNAs encoding both *lacZ* and MKUZDN (B) were injected into one cell of a 2–4 cell stage *Xenopus* embryo, and embryos were fixed at the neural plate stage. *lacZ* RNA served as a tracer to follow the distribution of the injected RNAs and was revealed by a *lacZ* substrate that gave a red color upon histochemical reaction. The *lacZ* negative side indicated the uninjected side and served as a control. Injection of RNA encoding MKUZDN resulted in extra *N-tubulin* positive cells as compared to the uninjected side (B) or to embryos injected with *lacZ* RNA alone (A). Extra neurons were observed in 72% ($n = 156$) of the embryos injected with MKUZDN. Note that these extra *N-tubulin* positive cells were confined to domains of primary neurogenesis and were not observed at ectopic positions.

(C), (D), (E), and (F) show expression patterns of a *Xenopus kuz* gene (*Xkuz*, partial sequence shown in Figure 1A) at different stages. (C) and (D) show the uniform expression of *Xkuz* in stage 13 embryos (dorsal view in [C] and lateral view in [D]). The ventral vegetal cells were not stained (D), most likely due to the low penetrance of probes into these cells. In older embryos, *Xkuz* continues to be widely expressed, with an elevated level in neural tissues (E and F).

an activated form of Notch (reviewed in Artavanis-Tsakonas et al., 1995) under the heat shock promoter ($hs-N^{act}$) at early pupal stages (7–9 hours APF) leads to the loss of microchaetes on the notum (Figure 4D); the opposite phenotype, extra microchaetes, is seen in loss-of-function *kuz* mutant clones (Figure 4E). We focused on microchaetes since the SOPs for these bristles are generated more synchronously than those of the macrochaetes (Hartenstein and Posakony, 1989; Huang et al., 1991) and thus a single pulse of heat shock at 7–9 hrs APF results in the reproducible loss of microchaetes on the notum in $hs-N^{act}$ flies. If *kuz* acts genetically downstream of *N*, then the combination of N^{act} and *kuz* should display the *kuz* phenotype of extra microchaetes. Conversely, if *kuz* acts genetically upstream of *N*, then the combination of N^{act} and *kuz* should display the N^{act} phenotype of missing microchaetes. We observed that the

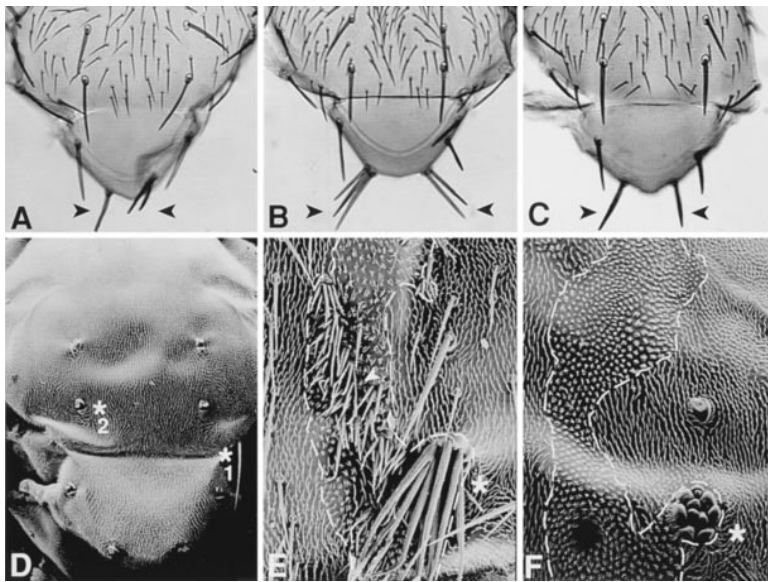


Figure 4. Genetic Interactions between *kuz* and *Notch*

(A–C) The phenotype of a weak KUZDN transgene was examined in wild-type (A), *N*⁵³¹⁷/+ (B) and +/Dp(1;Y)w⁺303 (C) backgrounds. *N*⁵³¹⁷ is a strong loss-of-function *N* allele (Karim et al., 1996). Dp(1;Y)w⁺303 (Lindsley and Zimm, 1992) is a duplication that includes the *N* locus, and thus +/Dp(1;Y)w⁺303 flies contain an extra copy of the *N* gene. Note the enhancement of extra bristle phenotype in (B) and the suppression of this phenotype in (C) (arrowheads).

(D–F) A 60 min heat shock at 37°C administered 7–9 hr APF resulted in a complete loss of microchaetes on the notums in flies bearing *hs-N*^{act}. Since the heat shock is delivered after the SOPs for macrochaetes are selected, this treatment does not abolish the SOPs for macrochaetes that are determined at an earlier time. Instead, it results in a shaft-to-socket transformation by interfering with a cell-fate decision that occurs at this time in developing macrochaetes. This transformation is not fully penetrant such that while

some macrochaetes are transformed into double sockets (asterisk 2 in [D]), others remain untransformed (asterisk 1 in [D]). (E) shows a *kuz* mutant clone on the notum marked by the cuticle marker *ck* (*ck* results in the secretion of multiple cytoplasmic extensions or hairs instead of a single hair from each epidermal cell). The clone boundary is outlined. Note the presence of clusters of macrochaetes (asterisk) and microchaetes (arrowhead) in *kuz* mutant clones. However, in *kuz* mutant clones that expressed *N*^{act} at 7–9 hrs APF (F), we never observed microchaete clusters as seen in *kuz* mutant clones. Occasionally, we observed one single microchaete in a large clone, presumably due to the incomplete penetrance of *N*^{act} expression. Note that while induction of *N*^{act} expression at this developmental time completely abolished microchaetes, macrochaetes clusters (asterisk in [F]) were still formed in the *kuz* mutant clones, except that often the shafts of the macrochaetes were transformed into sockets, as seen when *N*^{act} is expressed in a *kuz*⁺ background (D). Thus the phenotypes induced by expression of *N*^{act} are the same in a *kuz*⁺ or a *kuz*[−] background.

combination of *N*^{act} and *kuz* displayed the *N*^{act} phenotype (Figure 4F), suggesting that *kuz* acts genetically upstream of *N*. This result is compatible with KUZ acting upstream of Notch in the same biochemical pathway. However, our genetic experiments cannot rule out the possibility that KUZ and Notch act in parallel pathways.

We observed dosage-sensitive genetic interactions between *kuz* and *N*, indicating that the levels of activity of *kuz* and *N* are tightly balanced. We took advantage of a weak *dpp*-KUZDN transgene that resulted in an average of 3 posterior scutellar bristles (Figure 4A) instead of the 2 seen in wild-type. While heterozygous *N* mutants have normal numbers of posterior scutellar bristles (data not shown), this genetic background dramatically enhanced the phenotype resulting from the weak *dpp*-KUZDN transgene such that an average of 5.2 bristles (*n* = 50) were observed (Figure 4B). Furthermore, in flies that carry an additional copy of the *N* gene, the extra bristle phenotype resulting from this KUZDN transgene is completely suppressed such that 2 bristles were observed (Figure 4C, *n* = 50). This intricate balance between their activities suggests that *kuz* and *N* are closely linked in a common biological process.

kuz Is Required for the Proteolytic Processing of Notch

The genetic interactions between *kuz* and *N*, together with the similarity of their loss-of-function phenotypes and the previous clonal analyses indicating that *kuz* and *N* function in the same cell, prompted us to investigate whether KUZ is required for the proteolytic processing

of Notch. First, we examined if perturbation of KUZ function in *Drosophila* Schneider 2 (S2) cell cultures would affect Notch processing. S2 cells do not express any endogenous Notch protein (Fehon et al., 1990), but do express high levels of *kuz* mRNA (data not shown). Upon transfection of a full-length *N* construct, the monoclonal antibody C17.9C6, which was raised against the intracellular domain of Notch, can detect full-length Notch (about 300 kDa) and C-terminal fragments of about 100 kDa (Fehon et al., 1990; Figure 5, lane 1). We reasoned that if *kuz* is involved in generating this 100 kDa species in S2 cells, then expression of KUZDN might interfere with this proteolytic event. Indeed, expression of KUZDN nearly abolished the 100 kDa species in S2 cells, while the 300 kDa species was not greatly affected (Figure 5, lane 2), suggesting that *kuz* is required for the Notch processing. Consistent with our results in transgenic flies that overexpression of full-length KUZ did not perturb neurogenesis, transfection of a full-length KUZ construct did not affect Notch processing in S2 cells (Figure 5, lane 3).

Next, we performed similar experiments in developing imaginal discs. As described earlier, in transgenic flies containing KUZDN under the control of the heat-shock promoter, a one hour heat shock at the third instar larval stage resulted in extra bristles on the notum (Figure 2K). The same heat-shock regime also resulted in notches on the wing blade and extra photoreceptors in the eye (data not shown). We followed the status of Notch processing in the wing and eye imaginal discs after the induction of KUZDN in these animals. As in transfected S2 cells, MAb C17.9C6 normally detects a 300 kDa and

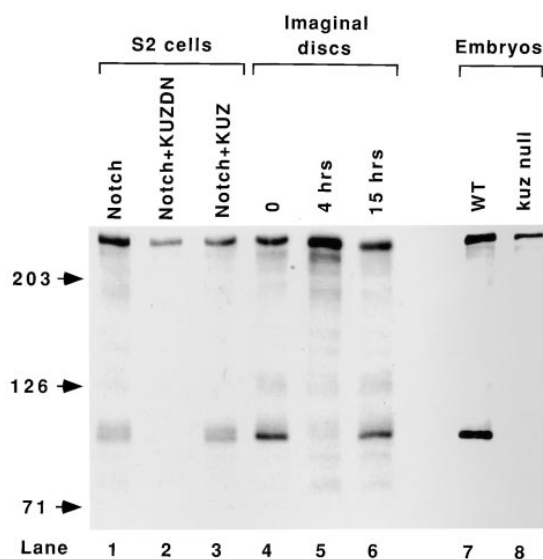


Figure 5. *kuz* Is Required for the Proteolytic Processing of Notch. Protein extracts from S2 cells (lane 1–3), imaginal discs (lane 4–6), and embryos (lane 7 and 8) were separated on 6% SDS–PAGE, transferred to nitrocellulose, and visualized with C17.9C6, a monoclonal antibody against the intracellular domain of Notch (Fehon et al., 1990). Besides the full-length Notch at about 300 kDa, this antibody also detected processed Notch proteins of about 100 kDa in S2 cells transfected with a full-length Notch construct (lane 1), imaginal discs (lane 4), and embryos (lane 7). Expression of KUZDN specifically abolished the 100 kDa species in S2 cells (lane 2), while the full-length KUZ did not have any effect (lane 3). Similarly, in imaginal discs following the induction of KUZDN in larvae carrying the *hs/KUZDN* transgene with a 60 min heat shock at 37°C, the 100 kDa species gradually disappeared such that by 4 hr after induction, the 100 kDa species was almost undetectable, while the 300 kDa species accumulated to a higher level (lane 4, no heat shock; lane 5, 4 hr post heat shock). The level of the 100 kDa species was gradually restored to the wild-type level (lane 6, 15 hr post heat shock). Lastly, in *kuz* null embryos, the 100 kDa form is not detectable (lane 8), while this form constitutes about half of the total Notch protein in wild-type embryos (lane 7).

a 100 kDa Notch species in protein extracts of the third instar imaginal discs (Figure 5, lane 4). After the induction of KUZDN by one hour of heat shock, the 100 kDa species gradually disappears; by 4 hours after induction, the 100 kDa species is almost undetectable, while the 300 kDa species has accumulated to a higher level (Figure 5, lane 5). By 15 hrs after the heat shock, the 100 kDa species is restored to wild-type levels (Figure 5, lane 6), presumably reflecting the decay of the KUZDN protein synthesized in response to the heat shock. The correlation between the reduction of the 100 kDa species upon KUZDN expression (Figure 5) and the resulting neurogenic phenotypes in imaginal tissues (Figure 2K) provides evidence for the functional significance of the 100 kDa Notch form detected in vivo.

Finally, we examined Notch processing in *kuz* null mutant embryos. Since *kuz* is known to have a maternal contribution (Rooke et al., 1996), we generated germline clones to obtain embryos lacking all KUZ function. We found that while MAb C17.9C6 detects a 300 kDa and a 100 kDa species in wild-type embryos, only the 300

kDa species is detected in *kuz* null embryos. This observation further supports our conclusion that KUZ controls proteolytic processing of Notch in vivo.

Discussion

In this study, we provide genetic and biochemical evidence that *kuz* is required for Notch processing in vivo, and furthermore, that this processing is important for Notch function. The dosage-sensitive genetic interactions between *kuz* and *N* suggest that *kuz* and *N* are involved in a similar signal transduction pathway. The phenotype observed in flies carrying a constitutively activated Notch and a loss-of-function *kuz* mutation indicates that *kuz* acts genetically upstream of *N*. We show that Notch processing is abolished in *kuz* null embryos and in imaginal discs or tissue culture cells expressing a dominant-negative KUZ construct. These results demonstrate that *kuz* is required for the proteolytic cleavage of Notch that generates the 100 kDa C-terminal fragment in vivo. The simplest explanation is that *kuz*, which encodes a metalloprotease, directly processes Notch to generate the 100 kDa species, although further biochemical experiments are required to rule out alternative explanations such as a proteolytic cascade. In addition, the correlation between the neurogenic phenotypes observed in *kuz* null embryos or in imaginal discs overexpressing KUZDN and the absence of Notch processing in these contexts suggests that this processing event, detected in worms, flies, and vertebrates, is required for Notch function.

While our studies indicate that Notch is a substrate for KUZ, it is important to note that the phenotype of the *kuz* mutation is not fully explained by its affect on Notch processing. For example, the embryonic neurogenic phenotype of a *kuz* null mutant is more severe than that of a *N* null (Rooke et al., 1996). There are likely to be additional proteolytic substrates for KUZ; for example, the substrate(s) that is responsible for the axonal extension phenotype of *kuz* (Fambrough et al., 1996; this study) remains to be determined. Moreover, KUZ has domains, in addition to the protease domain, that are known to be important for the function of other ADAM family members. While our work does not reveal a role for these domains in lateral inhibition, it is likely that they play a role in other processes.

Although our experiments demonstrate that *kuz* is required for a particular proteolytic processing of Notch, they did not address whether this processing occurs after ligand binding or represents a maturation step required to make a competent Notch protein, in a manner similar to the maturation of the Sevenless receptor (Simon et al., 1989). Based on the findings that engineered forms of the Notch proteins that delete the extracellular domain, or both the transmembrane and the extracellular domains, result in constitutively active receptors, some models of Notch signaling propose that the Notch protein is processed on the intracellular side to release its cytoplasmic domain upon ligand binding (reviewed in Artavanis-Tsakonas et al., 1995). The particular proteolytic cleavage we are concerned with in our study is distinct from this intracellular cleavage. The KUZ-mediated cleavage most likely occurs in the extracellular

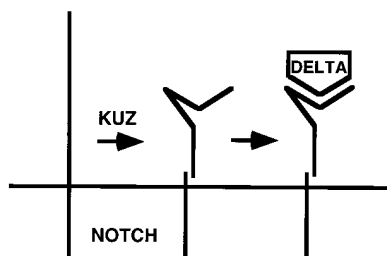


Figure 6. A Model for the Synthesis of Notch Proteins
See text for details.

domain of Notch and does not seem to be the result of receptor activation upon ligand binding. First, the 100 kDa species is detected in S2 cells transfected with a full-length Notch construct (Fehon et al., 1990; this study). It is unlikely that the 100 kDa species is a product of Notch activation upon ligand binding, since these cells do not express any known Notch ligand. Second, the 100 kDa species constitutes about half of the total Notch protein in *Drosophila* imaginal discs or embryos detected with an antibody against the intracellular domain (see Figure 5, lanes 4, 6, and 7) and represents the only Notch protein detected with a similar antibody in certain mammalian cells (Zagouras et al., 1995). It seems unlikely that they all represent activated Notch proteins. Third, studies of vertebrate Notch proteins have suggested that the proteolytic cleavage responsible for generating the 100 kDa species occurs on the extracellular domain of Notch and does not by itself lead to activation of Notch proteins (Aster et al., 1994; Kopan et al., 1996).

Since the metalloprotease domain of KUZ is extracellular, we propose that KUZ protease processes Notch on the extracellular domain to generate an N-terminal extracellular fragment and the C-terminal 100 kDa fragment containing the transmembrane and the cytoplasmic domain (Figure 6). These two fragments might then be tethered together to function as a competent Notch protein, analogous to the maturation of the Sevenless receptor (Simon et al., 1989). This is consistent with experiments in *C. elegans* showing that the N- and C-terminal fragments of GLP-1 protein can be copurified (Crittenden et al., 1994). It is also consistent with the known activity of another ADAM protein TACE, which carries out an extracellular cleavage on its substrate, the TNF- α precursor (Black et al., 1997; Moss et al., 1997). The processing of Notch need not happen on the cell surface; it could take place in the endoplasmic reticulum, the Golgi apparatus, or secretory vesicles during the maturation of Notch. Indeed, Blaumueller et al. (1997 [this issue of *Cell*]) present evidence that Notch is cleaved in the trans-Golgi as the protein traffics toward the plasma membrane and that only the cleaved forms of Notch are found on the cell surface. The biochemical role that this proteolytic processing plays in Notch function is unclear at present, but our genetic data suggest that Notch needs to be cleaved to be a functional receptor. The binding of ligands such as Delta to the competent receptor might dissociate the N- and C-terminal fragments such that the membrane-spanning

C-terminal fragment could be further processed on the intracellular side by an unidentified protease to release the cytoplasmic domain of Notch, which might translocate to the nucleus, as suggested by some investigators. In this regard, it is worth noting that while intracellular processing of Notch upon ligand binding has yet to be demonstrated, a truncated activated Notch construct resembling the C-terminal 100 kDa fragment is known to be processed on its intracellular side to release its cytoplasmic domain in 3T3 cells (Kopan et al., 1996).

The ADAM family is a recently defined family of transmembrane proteins that contain both a disintegrin-like and metalloprotease-like domain (reviewed in Wolfberg et al., 1995). Although cell biological and biochemical studies have implicated ADAM family members in diverse developmental processes such as fertilization (fertilins), myoblast fusion (meltrin), and inflammatory response (TACE), the molecular mechanisms of ADAM functions are largely unknown. *kuz* represents the first ADAM family member in any species whose loss-of-function phenotype has been characterized, providing us the opportunity to study an ADAM gene in a well defined genetic system. It is likely that different ADAM family members will share signaling mechanisms, and that studies of KUZ can provide insights into the functions of ADAM proteins in general. Our studies on KUZ provide a general scheme for engineering dominant-negative forms of ADAM proteins and could be applied to other ADAM genes for probing their biological functions. It is worth noting that while all ADAMs possess a disintegrin-like and a metalloprotease-like domain, some ADAMs lack a consensus active site in the metalloprotease domain. These "protease dead" ADAMs therefore resemble dominant-negative forms of KUZ described in this study and might function as endogenous inhibitors of certain biochemical processes.

Experimental Procedures

Plasmid Constructs

We initially used the pGMR vector (Hay et al., 1994) to express full-length KUZ and several N- and C-terminal deletion constructs in the eye. These constructs include 1, 2, 3, 4, and 7. Upon identification of 7 as a dominant-negative form (KUZDN), we then used another expression vector, pDMR, to express constructs 1, 4, 5, 6, 7, 8, and 9. The pDMR vector utilizes the *dpp* disc-specific enhancer to drive gene expression in multiple tissues including the wing and the notum. pDMR was constructed by the following steps. First, the heat shock-responsive element in Casperhs (Piotto, 1988) was removed to yield Casperhs-1. A 4.3 kb *dpp* disc-specific enhancer (Staehling-Hampton et al., 1994) was inserted upstream of the *hsp70* basal promoter in Casperhs-1 to yield pDMR (*dpp*-mediated reporter). Construct 7 (KUZDN) was also cloned into pUAST (Brand and Perrimon, 1993) and pCasperhs to generate UAS/KUZDN and hs/KUZDN, respectively. A *rough* enhancer element (Heberlein et al., 1994) was then inserted into hs/KUZDN to generate *rough*/KUZDN. Constructs 1 (full-length KUZ) and 7 (KUZDN) were also cloned downstream of the metallothionein promoter in pRMHa-3, an S2 cell expression vector (Bunch et al., 1988). The nucleotide coordinates of constructs 1–9 are as follows, using the same numbering as in GenBank accession no. U60591: 1 and 8, 723–5630; 2, 723–3578; 3, 723–3462; 4, 723–2757; 5, 1957–2757; 6, 1957–5630; 7 and 9, 2757–5630. Note that for all of the N-terminal deletion constructs, a DNA fragment (nucleotides 723–940) containing the signal peptide was provided at the 5' end. Site-directed mutagenesis was carried out using Stratagene's QuickChange system.

MKUZDN was generated by an N-terminal truncation that removes

the pro and catalytic domains of MKUZ. The rest of MKUZ (nucleotides 1395–2481 of GenBank accession no. AF011379) was ligated either to a DNA fragment (723–940, according to nucleotide coordinates in U60591) containing the signal peptide of *Drosophila* KUZ to generate MKUZDN-1 or to a fragment (nucleotides 1–191 of GenBank accession no. AF011379) containing the signal peptide of MKUZ to generate MKUZDN-2. MKUZDN-1 was subcloned into pDMR and pUAST for overexpression in *Drosophila*, and MKUZDN-2 was subcloned into a modified CS2+ vector (Turner and Weintraub, 1994) for RNA injection in *Xenopus* embryos (see below).

Characterization of *kuz* Homologs from Mouse and *Xenopus*

PCR primers corresponding to sequences of a rat gene similar to *kuz* (GenBank accession no. Z48444) were used to amplify a fragment from a mouse brain cDNA library. The sequences (5' to 3') of the PCR primers are: GAAGTTGGACATAACTTTGGATC and AATCCAGCCATTAGCATGATCAGG. The PCR product was then used to screen oligo(dT) and random-primed cDNA libraries from the mouse PCC4 cell line (Stratagene). Two overlapping cDNA, *mkuz2* and *mkuz3*, were characterized and sequenced, which together comprised the whole coding region. *mkuz2* extends from nucleotide 353 to 2481, and *mkuz3* extends from 1 to 1259 (GenBank accession no. AF011379).

Xenopus kuz was cloned by PCR using the degenerate primers CA(C/T)AA(C/T)TT(C/T)GGNTCNCNC(A/C/T)GA (XK1) and AANA(C/G/A)TC(G/A)CA(G/A)TANCC (XK4) that correspond to *Drosophila* KUZ sequences HNFGSPHD and GYCDVF, respectively. First-strand cDNA from stage 18 *Xenopus* embryos (gift of Francesca Mariani and Richard Harland) was used as template in a standard PCR reaction with an annealing temperature of 50°C. A PCR product of expected size was purified and used as template for another PCR reaction using a nested primer GA(G/A)GA(G/A)TG(C/T)GA (C/T)TG(C/T)GG (XK3), corresponding to *Drosophila* KUZ sequence EECDCG, and XK4. The PCR product was subcloned into Bluescript and sequenced. Anti-sense RNA was used as a probe for whole-mount in situ hybridization of *Xenopus* embryos according to standard procedures (Harland, 1991).

For RNA injections in *Xenopus* embryos, MKUZDN-2 was synthesized in vitro using SP6 RNA polymerase from a CS2+ vector. Nuclear *lacZ* RNA was synthesized from plasmid pSP6nucβGal (gift of R. Harland). MKUZDN RNA (500 pg), together with *lacZ* RNA (100 pg), was injected into one blastomere of *Xenopus* embryos at 2–4 cell stage. *lacZ* RNA was also injected alone as a control. Embryos were fixed at the neural plate stage and stained with Red-Gal (Research Organics, Inc.). Embryos were then processed for in situ hybridization with a neural-specific β-tubulin probe (gift of Chris Kinter).

Drosophila Genetics

For epistasis between *kuz* and *Notch*, an activated *N* construct containing only the cytoplasmic domain of Notch (*N^{act}*) under the control of the heat-shock promoter (ITM3A insertion on the X chromosome, from Lieber et al. [1993]) and a null *kuz* allele *e29-4*, which deletes ~2.4 kb near the 5' end of the *kuz* gene, including DNA in the promoter region and the first and second exons (Rooke et al., 1996), were used. Flies of the genotype ITM3A/+; *e29-4 ck* FRT40A/+ were crossed to hsFlp/Y; FRT40A. The progeny from such a cross were subjected to a 1-hr heat shock at 38°C 24–48 hr after egg laying to induce *kuz* mutant clones and another 1-hr heat shock at 7–9 hr APF to induce the expression of *N^{act}*. Adult flies were processed for scanning electron microscopy and the clones identified by the cell-autonomous *ck* epidermal hair marker as in Rooke et al. (1996). The clones shown in Figures 2C and 2D were also marked with *ck* (not shown in the figure).

kuz germline clones were generated as in Rooke et al. (1996). Females bearing germline clones were mated to *e29-4/CyO* males. *kuz* null embryos lacking both maternal and zygotic contribution can be distinguished from *kuz* maternal null embryos rescued with one zygotic copy of *kuz* at late embryonic stages, since *kuz* null embryos fail to develop any cuticle while paternally rescued embryos develop some cuticle structures. *kuz* null embryos were hand-picked for making protein extracts.

Protein Extracts and Immunoblotting

Approximately 2×10^6 S2 cells, 50 embryos, or imaginal discs from 16 third instar larvae were used for each extraction. These materials were homogenized and incubated for 20 min on ice in 90 μl of buffer containing 10 mM KCl, 20 mM Tris (pH 7.5), 0.1% mercaptoethanol, 1 mM EDTA plus protease and phosphatase inhibitors (leupeptin, aprotinin, PMSF, and sodium vanadate). Supernatant was collected after a low-speed spin of 2000 rpm for 5 min. Supernatant (12 μl) was run on a 6% SDS-polyacrylamide gel. Blotting, antibody incubation, and chemiluminescent detection using the ECL kit were as described in Fehon et al. (1990).

Acknowledgments

Correspondence regarding this paper should be addressed to G. M. R. We thank Elaine Kwan and Chris Suh for technical assistance, Jose De Jesus, Francesca Mariani in Richard Harland's laboratory for advice and help with the *Xenopus* experiments, Richard Harland and Chris Kintner for *Xenopus* probes, Toby Lieber and Michael Young for activated Notch transgenic flies, and Spyros Artavanis-Tsakonas for Notch antibodies. Richard Harland, Marc Therrien, Elizabeth Chen, Mike Brodsky, and Corey Goodman provided useful comments on the manuscript.

Received April 29, 1997; revised June 18, 1997.

References

- Artavanis-Tsakonas, S., Matsuno, K., and Fortini, M.E. (1995). Notch Signaling. *Science* 268, 225–232.
- Aster, J., Pear, W., Hasserjian, R., Erba, H., Davi, F., Luo, B., Scott, M., Baltimore, D., and Sklar, J. (1994). Functional analysis of the *TAN-1* gene, a human homolog of *Drosophila Notch*. *Cold Spring Harbor Symp. Quant. Biol.* 59, 125–136.
- Black, R.A., Rauch, C.T., Kozlosky, C.J., Peschon, J.J., Slack, J. L., et al. (1997). A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature* 385, 729–733.
- Blaumueller, C.M., Qi, H., Zagouras, P., and Artavanis-Tsakonas, S. (1997). Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. *Cell*, this issue, 90, 281–291.
- Blobel, C.P., Wolfsberg, T.G., Turck, C.W., Myles, D.G., Primakoff, P., and White, J.M. (1992). A potential fusion peptide and an integrin ligand domain in a protein active sperm-egg fusion. *Nature* 356, 248–252.
- Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, 401–415.
- Bunch, T.A., Grinblat, Y., and Goldstein, L.S.B. (1988). Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucleic Acids Res.* 16, 1043–1061.
- Campos-Ortega, J.A. (1993). Early neurogenesis in *Drosophila melanogaster*. In *The Development of Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1091–1129.
- Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995). Primary neurogenesis in *Xenopus* embryos regulated by a homologue of the *Drosophila* neurogenic gene *Delta*. *Nature* 375, 761–766.
- Crittenden, S.L., Tromel, E.R., Evans, T.C., and Kimble, J. (1994). GLP-1 is localized to the mitotic region of the *C. elegans* germ line. *Development* 120, 2901–2911.
- Fambrough, D., Pan, D., Rubin, G.M., and Goodman, C.S. (1996). The cell surface metalloprotease/disintegrin Kuzbanian is required for axonal extension in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 93, 13233–13238.
- Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A.T., and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci *Notch* and *Delta*, two EGF-homologue genes in *Drosophila*. *Cell* 61, 523–534.

Harland, R. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Meth. Cell Biol.* 36, 685–695.

Hartenstein, V., and Posakony, J.W. (1989). Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development* 107, 389–405.

Hartenstein, V., and Posakony, J.W. (1990). A dual function of the *Notch* gene in *Drosophila* sensillum development. *Dev. Biol.* 142, 13–30.

Hay, B.A., Wolff, T., and Rubin, G.M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* 120, 2121–2129.

Heberlein, U., Penton, A., Falsafi, S., Hackett, D., and Rubin, G.M. (1994). The C-terminus of the homeodomain is required for functional specificity of the *Drosophila rough* gene. *Mech. Dev.* 48, 35–49.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* 64, 1083–1092.

Howard, L., Lu, X., Mitchell, S., Griffiths, S., and Glynn, P. (1996). Molecular cloning of MADM: a catalytically active mammalian disintegrin-metalloprotease expressed in various cell types. *Biochem. J.* 317, 45–50.

Huang, F., Dambly-Chaudiere, C., and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*. *Development* 111, 1087–1095.

Jiang, W., and Bond, J.S. (1992). Families of metalloendopeptidases and their relationships. *FEBS Lett.* 312, 110–114.

Karim, F.D., Chang, H.C., Therrien, M., Wassarman, D.A., Laverty, T., and Rubin, G.M. (1996). A screen for genes that function downstream of Ras1 during *Drosophila* eye development. *Genetics* 143, 315–329.

Kopan, R., Schroeter, E.H., Weintraub, H., and Nye, J.S. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. *Proc. Natl. Acad. Sci. USA* 93, 1683–1688.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V., and Young, M.W. (1993). Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev.* 7, 1949–1965.

Lindsley, D.L., and Zimm, G.G. (1992). The genome of *Drosophila melanogaster* (San Diego, CA: Academic Press).

Moss, M.L., Jin, S.L.C., Milla, M.E., Burkhart, W., Carter, H.L., et al. (1997). Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor- α . *Nature* 385, 733–736.

Pirotta, V. (1988). Vectors: A Survey of Molecular Cloning Vectors and Their Uses. Rodriguez, R.L., and Denhardt, D.T., eds. (Boston, MA: Butterworths), pp. 437–456.

Rooke, J., Pan, D.J., Xu, T., and Rubin, G.M. (1996). KUZ, a conserved metalloprotease-disintegrin protein with two roles in *Drosophila* neurogenesis. *Science* 273, 1227–1231.

Simon, M.A., Bowtell, D.D.L., and Rubin, G.M. (1989). Structure and activity of the sevenless protein: a protein tyrosine kinase receptor required for photoreceptor development in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 86, 8333–8337.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Development* 109, 509–519.

Staehling-Hampton, K., Jackson, P.D., Clark, M.J., Brand, A.H., and Hoffmann, F.M. (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in *Drosophila* embryos induced by *decapentaplegic* but not *60A*. *Cell Growth Differ.* 5, 585–593.

Turner, D.L., and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* 8, 1434–1447.

Wolff, T., and Ready, D.F. (1993). Pattern formation in the *Drosophila* retina. In *The Development of Drosophila melanogaster*. M. Bate and A. Martinez-Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Press) pp. 1277–1325.

Wolfsberg, T.G., Primakoff, P., Myles, D.G., and White, J.M. (1995).

ADAM, a novel family of membrane proteins containing A Disintegrin And Metalloprotease domain: multipotential functions in cell-cell and cell-matrix interactions. *J. Cell Biol.* 131, 275–278.

Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y., and Fujisawa-Sehara, A. (1995). A metalloprotease-disintegrin participating in myoblast fusion. *Nature* 377, 652–656.

Zagouras, P., Stifani, S., Blaumueller, C.M., Carcangiu, M.L., and Artavanis-Tsakonas, S. (1995). Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc. Natl. Acad. Sci. USA* 92, 6414–6418.

GenBank Accession Numbers

The accession numbers for the *mkuz* and *xkuz* cDNA sequences reported in this paper are AF011379 and AF011380, respectively.